2nd QUARTERLY PROGRESS REPORT

POSSIBILITY OF GROWTH OF AIRBORNE MICROBES IN THE JOVIAN ATMOSPHERE

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This report describes our efforts to show growth activity of anaerobic bacteria in aerosolized particles suspended in anaerobic gas (N_2). In general, progress has been slow because of difficulties in culture production, but some evidence of spore generation in nitrogen has been obtained.

The first culture tested was Clostridium butyricum, which grew satisfactorily but cells were pleomorphic, swollen, and did not survive the aerosolization step. Clostridium butylicum grew too slowly for our purposes.

We decided to try Bacillus stearothermophilus, a facultative anaerobic spore-former that is quite heat resistant. At 55C this species grew well for about 10 hours in broth culture, but the acidic by-products of growth caused the pH to drop below growth limits. By the addition of calcium carbonate to the medium, we were able to achieve a sufficiently high number of cells (5×10^9/ml) to allow an aerosol test to be made. The results were what can be termed "standard" for a robust microbe in air; there was no loss of viability during a 3-day interval, no evidence of growth, and no evidence of "recuperation".

As a result of a welcome suggestion by Dr. Brewer, we have obtained a culture of C. perfringens and now have this species under cultivation. Prior to this, we consulted with Dr. Sacks, USDA, Albany, California, and Dr. Lumbard, CDC, Atlanta, Georgia, with respect to (a) possible pathogenicity of this species and (b) percentage of spore formation. The answers were that (a) although it can cause gas gangrene, it is as indigenous as staphylococci and the hazard via aerosols is trivial; (b) with the present state of the art, it is not possible to produce spore yield higher than 10^7/ml. Nonetheless, we intend to test the vegetative form of this anaerobic species for growth in the aerosol.

One species of spore-forming bacillus that is readily obtained in the spore form is B. subtilis; our laboratory has an adequate supply of frozen spore preparations. Although B. subtilis is an aerobe, we felt that testing this species under anaerobic, aerosol, conditions might yield some useful data while we were establishing suitable growth conditions for C. perfringens. A 40 ml portion of frozen B. subtilis spores was thawed, made up to 100 ml in distilled water that had been filtered via a 0.45μ Millipore filter, and centrifuged. Cells were resuspended and centrifuged in this manner an additional 4 times to remove as much excess material as feasible. The last precipitate was diluted to a volume of 20 ml, and heat-shocked for 20 minutes at 70°C to destroy vegetative forms; the final solution contained 5.3×10^9 spores/ml.

This suspension was atomized into two drums (31°C, 99+% RH); Drum I was filled with the spore suspension only; Drum II was simultaneously

filled via the "pinch-tube" noted in the previous report, with the spore suspension (in one atomizer) and with a solution of 1% glycerin and 0.5% tryptone in distilled water (in a second atomizer). The latter operation caused at least 50% of the spore particles to collide with nutrient particles. Samples from the aerosol were assayed before and after heat-shock. A decrease in the number of heat-shocked, viable cell numbers is presumptive evidence that spores have initiated processes leading to eventual germination and growth. Samples were also obtained in 1% formalin to be counted via the Coulter Counter.

Characteristics of spores alone

Results a' nown in Figures 1 and 2. There was no direct evidence of "germination" (in the sense of sensitivity to heat shock) during the first 6 hours aerosol time (Fig. 1), but there was an apparent increased, loss of viability after that time; followed by an increase. The word "apparent" is used to direct attention to a subtle measure of biological activity frequently found in other situations, and which has been termed "recuperation" (1). This is a situation in which the number of viable cells decrease as if death were occurring, followed by a period wherein the number of viable cells increases, sometimes at a rate greater than the growth rate under ideal conditions. This phenomenon cannot be growth unless the number apparently alive exceeds the number of cells initially present, or in the case of an aerosol, the maximal number that could be airborne when adjustment is made for loss of particles by fall-out. Recently, it has been shown that, contrary to popular belief, spores are not physiologically inactive, and they do undergo a rhythmic "recuperation" phenomenon similar to vegetative cells (2). It

is reasonable to assume that the process seen here is a similar circumstance.

Note that after 48 hrs there were almost as many viable cells remaining as would have been expected had no death occurred and if the particulate fall-out rate as determined by the Coulter Counter were correct, yet if the initial death rate were extrapolated to 48 hours, no viable cells would have remained. Also note that some germination appeared to have occurred, although it was not significant until after 24 hours.

We conclude from these data that washed spores of an aerobic species can initiate the process of germination in the airborne, anaerobic environment, although the process is slow.

<u>Characteristics of spores in contact with nutrients</u>

There was significant germination within 2 hours (Fig. 2) in a "burst", and again at 5 hours, and the same "recuperation" occurred as with washed spores alone, except that it happened prior to 24 hours. The two bursts noted are significant at the .01 probability level; i.e., the difference is greater than 3 standard deviations from the mean difference between duplicate assays.

The fact that some coagulation had occurred is illustrated by contrasting the particulate fall-out rates of the two aerosols; the half-life was 14 hours with spores alone and 10 hours for the mixed aerosol.

From these studies we concluded that at least the initial mechanisms leading to spore germination can operate in the anaerobic, airborne environment, and that it is highly probable that the process can continue, as reflected by the "recuperative" behavior, which is common with vegetative cells.

Cloud Chamber

Some control elements for the vertical chamber in which we intend to study air turbulence have arrived from the vendor and assembly has been started. The external air circulating system is almost completed and all containment systems have been leak-tested and the minor leaks have been repaired. We plan to start initial tests with the chamber about 15 December 1975.

Future Work

As soon as the growth characteristics of *C. perfringens* in a suitable medium have been ascertained (*ca*. 2 weeks), we will study the vegetative form in the anaerobic aerosol and, in view of the behavior of *B. subtilis* spores, we will not attempt to study the spore phase of *C. perfringens* until and unless we find evidence that some growth-related phenomenon is present in airborne cells.

We intend to test the idea that by placing microdroplets of fluid containing bacteria onto glass surfaces and exposing them to various nutritive vapors we can achieve a simple rapid screening technique to determine the

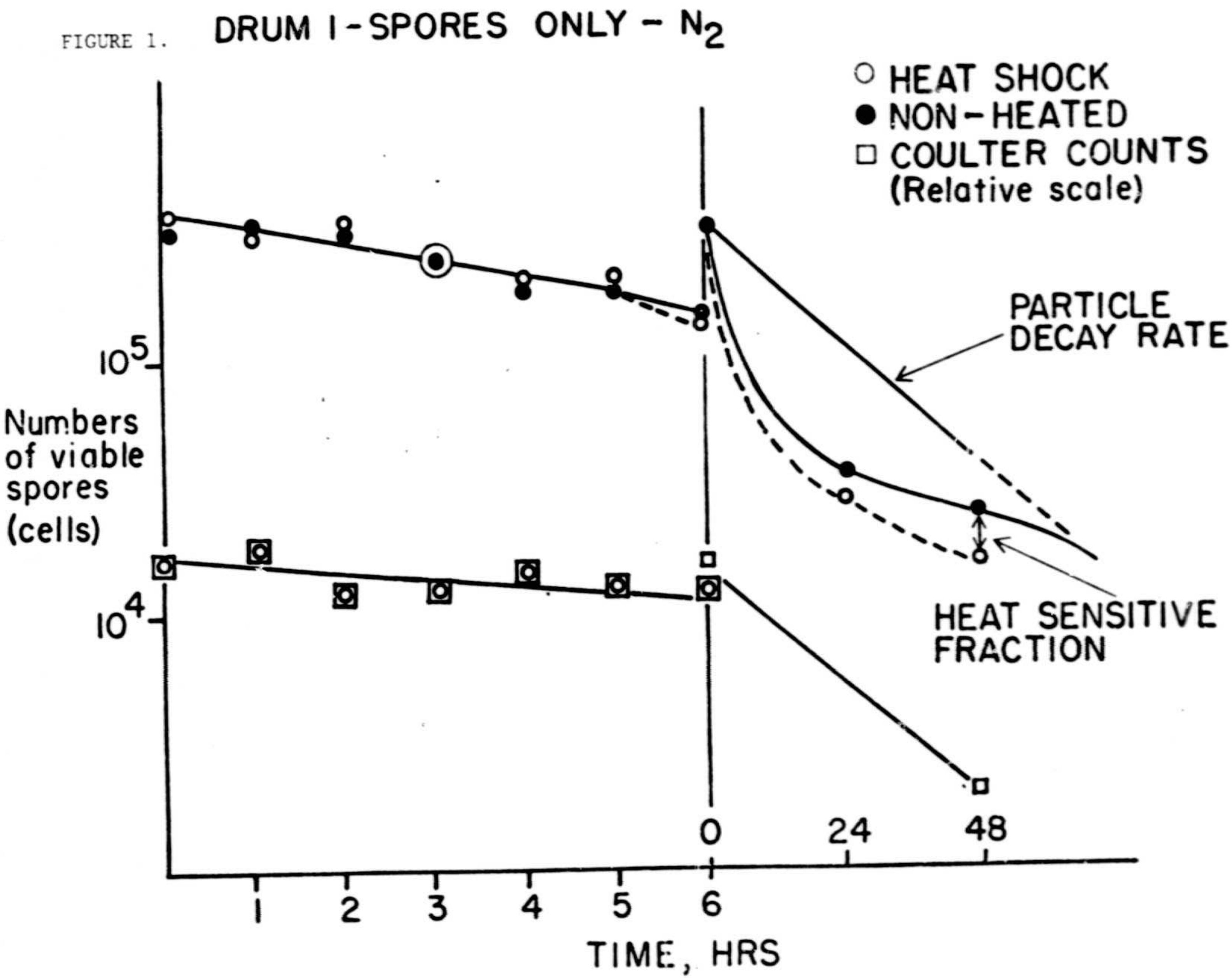
best combination (quality and quantity) of such materials needed to "feed" airborne microbes. If successful, the method will be more efficient than the direct use of microbes in aerosols.

REFERENCES

1. Dimmick, R.L. 1965. Rhythmic response of *S. marcescens* to elevated temperature. J. Bacteriol. 89:791-798.

2. Heckly, R.J. and J. DiMatteo. 1975. Rhythmic changes in dry heat resistance of *Bacillus subtilis* spores after rapid changes in pH. Appl. Microbiol. 29:565-566. (Appended)

FIGURE 1.



DRUM I-SPORES ONLY - N_2
HEAT SHOCK
NON-HEATED
COULTER COUNTS
(Relative scale)
PARTICLE
DECAY RATE
HEAT SENSITIVE
FRACTION
10^5
10^4
Numbers of viable spores (cells)
1
2
3
4
5
6
0
24
48
TIME, HRS

FIGURE 2. DRUM II - SPORE + MEDIUM - N_2